Amendments

In the Specification:

On page 1, please replace the first paragraph with the following:

--This application is a continuation of U.S. application no. 10/270,313, filed October 15, 2002, which is a continuation of U.S. application no. 09/851,425, filed May 9, 2001, now U.S. Pat. No. 6,608,108, which is a continuation-in-part of U.S. application no. 09/690,063, filed October 16, 2000, now U.S. Pat. No. 6,417,234, which is a continuation-in-part of U.S. application no. 09/418,594, filed October 15, 1999, now U.S. Pat. No. 6,214,874, all of which are hereby incorporated by reference.--

On page 7 at line 4, please replace the paragraph with the following:

--In a preferred embodiment, the compounds and compositions described herein are used in the treatment of HPV-induced tumors. HPV-induced tumors include in particular, but are not limited to, cervical, oral, penile and head and neck cancers that are associated with HPV infection. The method comprises local application of nordihydroguaiaretic acid derivatives, in particular ~~tetra-O-methyinordihydroguaiaretic~~ tetra-O-methylnordihydroguaiaretic acid (M_4N) and ~~tetraglycinal~~ tetraglycinyl nordihydroguaiaretic acid (G_4N), to cancerous and non-cancerous HPV-induced tumors.--

Beginning at page 8, line 22, to page 13, line 9, please replace the text with the following amended paragraphs:

~~Figure~~ FIG. 1. Structures of M_4N and G_4N.

~~Figure 2A.~~ FIG. 2 (Top) HPV-16 LCR showing region of E_6/E_7 promoter (pPV16P97) and the binding site for Sp1 protein. ~~Figure 2B.~~ (Bottom) The effect of M_4N on the E_6/E_7 promoter activity in C-33A cells. (Inhibition of E_6/E_7 promoter driven luciferase gene transcription by different concentrations of M_4N.)

~~Figures~~ FIGS. 3A-3C. Inhibition of Viral E_6 and E_7 RNA Transcripts by 40 μM M_4N.

Total RNA isolated from C_3 cells treated with either 40 μM M_4N or DMSO alone in growth media for 71 hours was subjected to relative RTPCR. The RTPCR samples were removed after increasing cycles of amplification and resolved on an agarose gel. The gel photographs (3A and 3B) indicate these cycles, the presence of (+) or absence (-) of M_4N in the growth media, and two digests of a pGMT vector used as size markers. The amplification map (2C) indicates the two expected size products of the amplification, resulting from the alternate splicing of the early viral RNA transcript.

~~Figure~~ FIG. 4A. Inhibition of C3 Cell Growth by M_4N.

~~Figure~~ FIG. 4B. Inhibition of C3 Cell Growth Following the Removal of M_4N.

~~Figures~~ FIGS. 5A-5B. Effect of M_4N on gene expression in C3 cells as examined by the GENE Assay analysis. 5A. GENE expressed in C3 cells after > 2 hours of DMSO treatment (C3 DMSO). 5B. GENE expressed in C3 cells after > 2 hours of M_4N treatment using DMSO as solvent (C3 M_4N).

~~Figures 6A-6B.~~ FIG. 6. Visual observations of tumor-bearing mice following M_4N treatment. ~~6A.~~ (Top) Mice bearing single tumors were treated with in situ injection of DMSO (#3) or M_4N (#7). *In situ* injection of M_4N was also made to one of the two tumors grown in mouse #9. ~~6B.~~ (Bottom) M_4N treated tumor (white scar) with untreated tumor from the same mouse, #9 as described in Table 2.

~~Figure~~ FIG. 7. Histopathology Effect of M_4N and M_4N/G_4N on Tumor Growth in Mice. First column from the panel presents the large size of tumors from mouse #4, 10, 12, following DMSO treatment (CON) as compared to the relatively small drug treated (M_4N or M_4N/G_4N) lesions from mouse #12, 10, 27 and 20 (M_4N). The subsequent photographs are examples of these tumors examined at 100X magnification (A, B, C, DMSO treated, D untreated, E, F, G, H, M_4N or M_4N/G_4N treated) mice (Table 1 and Table 2).

~~Figure~~ FIG. 8. HSV-1 replication in the absence of drugs (HSV-C, HSV-SC), in the presence of ineffective drugs ($ABDS_1$ ["HSV-$ABDS_1$"], $ABDS_2$ ["HSV-$ABDS_2$"])and in the presence of effective drugs (M_4N ["HSV-4N"] and ACV ["HSV-ACV"]).

~~Figure~~ FIG. 9. M_4N Causes Growth Arrest in Mammalian Cells. (a-d) C3, CEM-T4, C33a, and TC-1 cells were treated with different concentrations of M_4N. The number of cells present at the initiation of the experiment is indicated as Day 0. After three days the number of viable cells were counted and plotted versus the M_4N concentration. (e) C3 cells were split into T-25 flasks with 5 X 10^3 cells per flask and given either M_4N in 1% DMSO in media or 1% DMSO in media alone (first media change). After 3 days, one-half of the M_4N treated cells were given fresh media containing only 1% DMSO (M-D), while the rest of the cells were given fresh media with the same conditions (second media change). The cells were counted daily and plotted versus the time of treatment.

~~Figure~~ FIG. 10. Cells Treated With M_4N Arrest in G2/M. C3 cells (a), C33a cells (b), CEM-T4 cells (c), and TC1 cells (d) were grown for three days in media containing either 1% DMSO or 1% DMSO with M_4N (M_4N). The cells were trypsinized, fixed with ethanol, stained with propidium iodide, and were subsequently analyzed by flow cytommetry. The data is displayed as number of cells (3-5 X 10^4 total cells) versus propidium iodide stain intensity. The indicated stages of the cell cycle are labeled and correspond to the relative cellular DNA compliment as determined by staining intensity.

~~Figure~~ FIG. 11. C3 Cells Treated With 40μM M_4N Demonstrate G2 Cell Structures. C3 cells were grown on coverslips for three days in media containing either 1% DMSO (Control) or 1% DMSO with 40μM M_4N (M_4N). Samples were fixed with ethanol and incubated with antibodies against α (green) and γ (orange) tubulin (a) or with the DAPI DNA stain (b). Cells were examined by fluorescence microscopy.

~~Figure~~ FIG. 12. CDC2 and Viral Oncogenes are Reduced by M_4N. C3 cells were grown

for different amounts of time (numbers are in hours) in media containing either 1% DMSO (D) or 1% DMSO with 40 μM M_4N (M). After the specified times, total protein or total RNA was isolated from the cells. Western blots (a - top two panels) were performed using antibodies against CDC2 or cyclin B with the same nitrocellulose filter. Kinase assays (a -bottom two panels) were performed, following immunoprecipitation with antibodies to cyclin B, by incubation with γ-^{32}P ATP and histone H1. The coomassie stain of the PAGE gel is included as control for loading. Kinase assays for 24 and 72 hour drug treatments were performed separately. Northern blots (b) were performed on total RNA extracts. Filters were incubated overnight with random-primed ^{32}P-labeled DNA for CDC2 or GAPDH, washed, and exposed to film for three days. The same filter was used to test CDC2 and GAPDH RNA. rtPCR analysis (c) was performed on total RNA extracts with primers hybridizing to regions within either HPV-16 E7 or GAPDH. Both primer pairs were used in the same reactions, and the products were analyzed by agarose gel electrophoresis.

~~Figure~~ FIG. 13. Electrophoretic mobility shift assay (EMSA) of G_4N interaction with the HIV Sp1-binding sites (-87 to -49). (A) G_4N inhibition of Sp1-167D binding to ^{32}P labeled HIV Sp1 DNA template. Lane 1, template alone; lane 2, template plus 0.1 μg Sp1-167D; lanes 3-9, template incubated with increasing concentrations of G_4N (0.25 to 1.75 mM prior to the addition of 0.1 μg Sp1-167D. (B) G_4N displacement of Sp1-167D bound to HIV template. Lane 1, template alone; lane 2, template plus 0.1 μg Spl-167D plus 100-fold excess of unlabeled template; lane 3, template plus 0.1 μg Spl-167D; lanes 4-10, Spl/DNA complex challenged with increasing concentrations of G_4N (0.25 to 1.75 mM); lane 11, template incubated in reaction buffer containing 1.75 mM G_4N. (C) Sp1-167D displacement of G_4N bound to template. Lane 1, template alone; lanes 2-4, template plus increasing amounts of Sp1-167D (0.075, 0.150, 0.300 μg); lanes 5-8, template incubated in reaction buffer containing 1.2 mM G_4N followed by challenge with increasing amounts of Sp1-167D (0.075, 0.150, 0.300 μg), lane 8 received no Spl-167D. (D) Plot of diminishing Spl-167D/DNA complex band intensities in response to

increasing concentrations of G_4N used in (A) ---•--- and (B) —•—. The gels used were 5% non-denaturing polyacrylamide with each lane receiving 5 μl of each reaction volume as described in experimental section and Ref. [1].

~~Figure~~ FIG. 14. Inhibition of HIV Tat-regulated transactivation in Cos cells by G_4N.

~~Figure~~ FIG. 15. SIV production with presence of G_4N. 10^7 174 x cells, a human T-cell lymphoma cell line, were mixed with a 24 hrs. harvest stock of SIV mac 239 (4 ng of p27) for two hours at 37. Cells were resuspended and 1 x 10^5 cells in 100 μl medium were added to each well of three 96-well plates. Various concentrations of G_4N from a freshly made stock were prepared and added to each of the six designed well. Culture supernatants were collected after four and eight days for viral production analysis. Viral production was assayed by a modified p27 capsid protein antigen capture ELISA as described in experimental section.

~~Figure~~ FIG. 16. Inhibition of HIV p24 antigen production in H9 cells by G_4N. Inhibition in percentage was calculated by comparing p24 level from an average of two duplicate cultures of G_4N treated and not treated H9 cells 9 days following viral infection with a AZT resistant HIV strain, HIV-1RTMF.

~~Figure~~ FIG. 17. RT-PCR Analysis of Survivin Gene Expression. (a) Top: Survivin gene expression in C3 cells treated with 40 μM M_4N for 24 hours and 72 hours, respectively (lanes 3 and 4) and in the untreated controls (lanes 1 and 2). Bottom: the corresponding GAPDH controls. Band intensities were quantitated with Scion Image. (b) Survivin RT-PCR product signals were normalized to those of the GAPDH controls and plotted.

~~Figure~~ FIG. 18. Drug concentration-dependent down-regulation of survivin protein. (a) C3 cells were incubated with various concentrations of M_4N for 72 hours and the total cell lysate was immunoblotted against survivin. (b) Relative band intensities were

quantitated by Scion Image and plotted against M_4N concentration.

~~Figure~~ FIG. 19. Immunoblot analysis of caspase-3 cleavage in C3 cells treated with M_4N for 72 hours. (a) Western blot of caspase-3 showed cleavage of the 32 KD procaspase-3 and the formation of the active 20 KD cleaved product. (b) Band intensities were quantitated and plotted against M_4N concentration.

Please replace the paragraph beginning at page 13, line 20, with the following paragraph:

--Standard Procedure for the Preparation of *meso*-1,4-Bis [3,4 - (dimethylaminoacetoxy) phenyl]- (2R,3S)- dimethylbutane ~~Hydrochloride~~ Chloride Salt Tetraglycinyl NDGA, G_4N. To a dichloromethane (250 ml) solution containing NDGA (12.8 g, 42.3 mmol, 1.0 equiv) and N,N, -dimethylglycine (26.2 g, 254 mmol, 6.0 equiv) were added DCC (52.4 g, 254 mmol, 6.0 equiv) and DMAP (2.32 g, 18.9 mmol, 1.0 equiv). The reaction mixture was stirred for 24 h under nitrogen at room temperature. After the reaction mixture was filtered, the solution was concentrated under reduced pressure. Acetone (250 ml) was then added into the reaction flask and the solution was bubbled with excess HCl(g). The water-soluble precipitate was dissolved in H_2O and re-precipitated twice at room temperature from acetone to give (1) (~~29, 2~~ 29.2 g, 36.8 mmol) as a white solid in 87% yield. Proton NMR spectra were obtained on a Varian Unity-400 (400 MHz) spectrometer by use of D_2O solvent and TSP as Internal standard. Carbon-13 NMR spectra were obtained on a Varian Unity-400 (400 Mhz) spectometer by use of D_2O as solvent. Carbon-13 chemical shifts are referenced to the TSP singlet (δ0.0 ppm).--

On page 15, at line 1, please insert the following replacement formula in Scheme 1:

~~1. Me_2NCH_2COOH~~ 1. Me_2NCH_2COOH--

A marked-up copy of the scheme is enclosed.

On page 17, please replace the paragraph beginning at line 13 with the following:

--~~meso-1,4Bis[3,4(dimethyleminoacetoxy)phe 3S-dimethylbutane~~

~~Hydrochloride~~ Meso-1,4-Bis[3,4-dimethylaminoacetoxy)phenyl]-(2R,3S)-dimethylbutane Chloride Salt (2). To a solution of NDGA (1, 12.81 g, 42.37 mmol, 1.0 equiv) and N,N-dimethylglycine (26.21 g, 254.2 mmol, 6.0 equiv) in dichloromethane (250 ml) was added DCC (52.45 g, 254.2 mmol, 6.0 equiv) and DMAP (5.176 g, 42.37 mmol, 1.0 equiv). The reaction mixture was stirred for 24 h under nitrogen at room temperature. After dicyclohexylurea in the reaction mixture was filtered off, the resultant solution was concentrated under reduced pressure. Acetone (250 ml) was then added into the residue and the resultant solution was bubbled with excess HCl (g). The precipitate was dissolved in water and re-precipitated twice by use of acetone at room temperature to give 2 (28.97 g, 36.86 mmol) as a white solid in 87% yield: ^{1}H NMR (D_20, 400 MHz)δ 0.78 (d, J = 6.0 Hz, 6 H. 2 x CH_3), 1.73 (m, 2 H. 2 x CH), 2.38 (dd, J=13.2, 9.6 Hz, 2 H. 2 x ArCH), 2.78 (dd, J = 13.2, 4.4 Hz, 2 H. 2 x ArCH), 3.03 (s, 24 H. 8 x CH_3N), 4.53 (s, 8 H, 4 x CH_2N), 7.22 (m, 4 H. 4 x ArH), 7.29 (d, J= 8.4 Hz, 2 H. 2 x ArH); ^{13}C NMR(D_20, 100 MHz) δ 18.11, 40.82, 41.73, 46.75, 59.59, 125.79, 126.58, 131.63, 140.66, 142.47, 146. 11, 167.84; IR (KBr) 3461 (br), 2963 (m), 1777 (s, C=O), 1620 (m), 1478 (m), 1377 (m), 1210(m), 1106 (m), 961 (w), 852 (w) cm^{-1}; MS (FAB) of (2-4 HCl) m/z (relative intensity) 643 (M+, 30), 600 (20), 558 (43), 515 (20), 473 (42), 430 (13), 388 (26), 185 (18), 93 (38),58 (100), 44 (22); HRMS (FAB) of (2 - 4 HCl) calcd for $C_{34}H_{50}N_4O_8$ 642.3628, found 642.3614; Anal. Calcd for $C_{34}H_{54}N_4O_8Cl_4$: C, 51.78; H. 6.90; N. 7.10; O.16.23. Found: C, 51.70; H. 6.85; N. 7.05; O. 16.21.
It will be appreciated that by suitable substitution of other N,N-dimethyl-substituted amino acids, additional amino acid substituted compounds of the invention can be synthesized.--

On page 18, at line 26, please replace the paragraph with the following amended paragraph:

--The results (shown in ~~Figure~~ FIG. 2) demonstrate that M_4N dramatically reduces Sp1 regulated transcription initiation at the HPV E_6/E_7 promoter in luciferase assay.--

On page 19, line 3, please replace the paragraph with the following amended paragraph:

--Inhibition of E_6/E_7 mRNA synthesis following M_4N treatment was measured by RT-PCR in cervical cell line C_3· Relative RT-PCR was performed with quantities of total cellular RNA standardized to the cell numbers counted. The RT-PCR product was analyzed on a 2% agarose gel. The results are shown in ~~Fig.~~ FIG. 3. The RT-PCR results indicated that the amplified cDNAs of the expected size for E7 (321 bp) and E6 (204 bp) were detected in the DMSO treated cells as early as cycle 22 of amplification. These same products were barely detectable in the drug treated RNA extracts following 30 cycles of amplification. No amplified products were detected for the no template PCR control or from total RNA extracts of the HPV16-negative C33a cell line.—

Beginning on page 19, at line 16, please replace 2 paragraphs with the following two amended paragraphs:

--HPV-16 transformed immortal mouse epithelial cells (C3 cells) were plated at a density of 10^5 cells per vial. After 24 hours, ½ of the vials were given growth media containing 40 μM M_4N dissolved in 1% DMSO while the other half were given growth media containing only 1% DMSO. The results are shown in ~~Figure~~ FIG. 4A. Within 24 hrs a difference in cell morphology between drug treated and control C3 cells was observed. The growth and division of the drug treated cells was markedly reduced in comparison to the untreated control, while the fraction of viable cells compared to the total cell count remained constant for both drug treated and DMSO only control cells. This indicates that M_4N dramatically reduces cell division.

The effect on C3 growth following removal of M_4N from the medium was also examined. C3 cells were plated at a density of 10^4 cells per vial. At time=0, 2/3 of the vials were given growth media supplemented with 40 μM M_4N in 1% DMSO. The remaining vials were given growth media containing only 1% DMSO. After 73 hours, ½ of the vials that had received M_4N in their growth media were washed and media containing only 1% DMSO was added. The other 2/3 of the cell vials were washed and replaced with the same media administered before. The results, shown in ~~Figure~~ FIG. 4B,

indicate that the rate of cell growth was not notably increased in M_4N treated sample following the change to drug-free media, indicating that M_4N continues to significantly reduce cell division even after its removal from the extracellular environment.--

On page 20, at line 10, please replace the paragraph with the following amended paragraph:

--Gene expression with 9600 gene arrays was studied (~~Fig.~~ FIG. 5). Five micrograms each of poly A^+ RNA from 72 hrs. M_4N (40 μm) treated (C_3 M_4N) and non-treated (C_3 DMSO) was used in a pair of human 9600 gene array hybridization study according to the procedure described in Genomics 51, 313-324 1998. The hybridization image was captured by a color video camera with a Nikon 55 mm AF micro Niko lens and digitized by a Macintosh LC630 computer. Such detection via enzyme substrate reaction of color-forming enzymes in either single or dual-color mode is reproducible and extremely sensitive (can detect <5 copies of transcript per cell with RNAs from 10^7 cells).--

On page 21, please replace the paragraph beginning at the third from the last line with the following:

--Thirty six C57bl-16 NCR mice were injected with 5 x 10^5 C3 cells between the shoulders on the backs of the mice. Twenty four of the mice developed tumors within 20 days. Daily injection (50 μl-100 μl of M_4N or M_4N/G_4N) (200 mg/ml M_4N in DMSO, 200mg/ml G_4N in PBS) showed profound effect in tumor growth in animals, as shown in Tables 1 and 2, ~~Fig.~~ FIGS. 6 and 7.--

On page 26, at line 3, please replace the paragraph with the following:

--The effects of M_4N and M_4N/G_4N on C3 tumor growth in mice are summarized in Tables 1 and 2 and ~~Figures~~ FIGS. 5 and 6. Table 1 shows the drug effect on C3 cell growth in mice carrying single tumors. The average weight of four excised tumors of the control group was 1.48 g while weights of lesions from M_4N treated and M_4N/G_4N

treated were 0.142 and 0.51 g respectively. Drug treated lesions consisted mainly of dried out necrotic cells (~~Fig.~~ FIG. 6). Tumors from the control group appeared homogenous and contained actively growing cells. Table 2 shows the drug effect on C3 tumor growth in mice carrying multiple tumors. In this study, drug was injected into one of the tumors. The average weight of untreated tumors was 1.77 g while that of M_4N treated lesions was 0.15 g. Similar results were obtained following M_4N/G_4N injection—the average weight of untreated tumors was 1.27 g, while that of the drug treated lesions was only 0.103 g. The body weight changes of all mice during the entire experimental period appeared insignificant (Tables 1 and 2).--

On page 29, please replace the two paragraphs beginning on line 6 with the following:

--Our previous research on M_4N indicated that it could inhibit viral transcription by deactivation of Sp1-dependent promoters. Many mammalian cell cycle genes also contain essential Sp1 promoters and M_4N may therefore block their transcription. This hypothesis was tested by examining the antiproliferative effect of M_4N on a number of different cell lines. Low concentrations (10 μM) of the parent compound, NDGA, have previously been shown to induce apoptosis in mammalian cells (24). This effect, however, can be circumvented by blocking one of the catechol oxygens or the addition of a hydrophilic group to NDGA (25). Increasing amounts of the NDGA derivative M_4N were tested on cultures of the HPV-16/ras transformed C3 cell line (26) to determine the optimal concentration required to inhibit proliferation (~~Figure 9a~~ FIG. 9A). The cells respond well to M_4N, ceasing division after 72 hours over the range of concentration from 40 to 60 μM. After three days at these concentrations the number of cells remained equal to the count at the initiation of treatment (day 0, ~~Fig~~ FIG. 9). A more modest reduction in cell growth was observed at lower concentrations of the drug and some cell death was seen at concentrations greater than 60 μM.

The antiproliferative effect of M_4N on the C3 cell line is not solely due to the drug's ability to deactivate the Sp1-dependent HPV-16 E6/E7 oncogene promoter, as similar growth inhibition was observed in the HPV-16 transformed TC-1 cell line whose

E_6/E_7 oncogenes are under control of a non-Sp1 dependent retroviral promoter(27) (~~Figure 9d~~ FIG. 9D). In addition, growth of the C33a cell line (~~Figure 9c~~ FIG. 9C), an HPV-negative human cervical cancer cell line, and the CEM-T4 line (~~Figure 9b~~ FIG. 9B), a human leukemia cell line (28), was also blocked by treatment with M_4N. In the four cell lines that were treated with the drug, nearly all (>95%) of the arrested cells were viable until the concentration of M_4N exceeded a "threshold" value (60 μM for C3 cells, 40 μM for TC-1 cells, etc.). Above these concentrations the percentage of viable cells decreases precipitously. Interestingly, arrested cells maintained >95% viability even after prolonged exposure to the drug. The C3 cells exhibited no increase in cell death after eight days of treatment with 40 μM M_4N (~~Figure 9e~~ FIG. 9E).--

Please replace the text from page 30, line 10 to page 35, line 5,with the following amended paragraphs:

--Once it was established that cells treated with M_4N cease proliferation yet remain viable, analysis of cellular DNA content and fluorescence examination of cell structures were used to determine the point in the cell cycle where the cells arrest. Cells exposed to M_4N for 72 hours demonstrated increased G2/M DNA content relative to the controls (~~Figure 10a-d~~FIG. 10A-D). The most extreme responses were seen from the C3 and CEMT4 cell lines, in which >90% of the cells show G2/M DNA content.

In order to distinguish between an arrest in G2 or a mitotic block, antibodies against α tubulin (green) and γ tubulin (red) were used to determine the status of the centrosomes in the C3 cell line following 72 hours M_4N treatment. As shown in ~~Figure 11a~~FIG. 11A, the centrosomes of M_4N treated cells are duplicated but still located next to each other in the nucleus of the cell. Since centrosomes separate during early prophase, it can be concluded that these cells have not begun mitosis. In contrast, the gamma tubulin staining of the control cells has the diffuse pattern characteristic of G1 or S phase (29). A lack of chromatin condensation in the M_4N treated cells was also observed with DAPI staining (~~Figure 12b~~FIG. 11B), additional evidence that the cells have not moved forward out of G2 phase (30).

Example 11

Production of CDC2 is Inhibited by 40 μM M_4N

Since progression of cells out of G2 is dependent on the production of the MPF, the status of its protein components was examined in C3 cells treated with 40 μM M_4N. Asynchronous cells were grown for 24 or 72 hours in media containing either M_4N in 1% DMSO, or 1% DMSO alone. The cells were harvested, and equal amounts of total cellular protein were analyzed by western blotting. A marked reduction in the amount of CDC2 was observed after 72 hours treatment with M_4N (~~Figure 12a~~FIG. 12A). However, levels of cyclin B, detected by stripping and reprobing the same membrane, were found to be unchanged. These results indicate that, under these conditions, the arrest is not likely a response to p53 since it has been shown that overexpression of p53 leads to a decrease in cyclin B (31, 32). Consistent with the results of the western analysis, CDC2 kinase activity was eliminated by 72 hours of M_4N treatment (~~Figure 12a~~FIG. 12A). These experiments support the view that the drug acts by inhibiting the production of the CDC2 protein, resulting in a loss of activity of the MPF.

Our previous studies demonstrating the ability of M_4N to block Sp1-dependent viral transcription suggest reduction of CDC2 mRNA levels as a possible mechanism for the decrease of CDC2 protein. This is consistent with the finding that the cyclin B protein, whose gene does not require Spl for its expression, is produced at normal levels while the CDC2 protein, whose gene has two essential Spl sites in its promoter, is substantially reduced in quantity. To test this hypothesis, northern blot analysis was performed on RNA harvested from C3 cells treated with 40 μM M_4N for 5 to 72 hours. As shown in ~~Figure 12b~~FIG. 12B, the amount of CDC2 mRNA is reduced after only 24 hours treatment with M_4N and nearly eliminated after 72 hours. Production of the non-Sp1 regulated housekeeping gene GAPDH was used as an RNA loading control, and its levels were not effected by 40 μM M_4N.

The use of the C3 cell line allows us an additional control for analysis of the mechanism of M_4N mediated cell cycle arrest since other Sp1-dependent gene promoters are also likely to be inhibited by M_4N treatment. This possibility was examined in C3

cells by analyzing the effect of M_4N on transcription from the Sp1 dependent HPV-16 E_6/E_7 promoter. rtPCR analysis of RNA isolated from C3 cells treated with 40 μM M_4N for 5 to 72 hours demonstrated a clear reduction in the levels of the E_7 transcript (~~Figure 12c~~FIG. 12C). GAPDH was again used as an internal control in this experiment, and its levels were unaffected by drug treatment. These results provide additional evidence that M_4N reduces the transcripts of Sp1 regulated promoters.

Example 12

Inhibition of Sp1-Binding Activity by G_4N in a Gel Mobility-Shift Analysis.

Sp1 family proteins induce bends toward the major groove of DNA upon binding (33). The zinc finger domain of the Sp1 protein is responsible for the binding of the GC Box sequence 5'-GGGGCGGGG-3'. From computational analysis, it was determined that G_4N, the aminoester derivative of NDGA, could form a stable complex with such a sequence in the major groove. To determine whether G_4N can serve as an Sp1 blocker as well as an Sp1 displacer, we performed Sp1/enhancer interaction studies in the presence or absence of G_4N by the gel mobility-shift analysis using only the DNB binding domain of Sp1 for testing. In the blocking experiment, different concentrations of G_4N were first incubated with ^{32}P-labelled DNA in the binding buffer for 30 min at 25°C. DNA binding domain of recombinant Sp1 protein (Sp1-167D) was next added and incubated for additional 30 min in the presence of a large excess of BSA protein. In the displacement study, the recombinant SP1-167D was first allowed to bind DNA, G_4N was then added at the second step of the incubation. The G_4N and Spl-167D concentrations and, the incubation and gel electrophoresis conditions were identical in both studies (experimental section). As shown in ~~Figure~~ FIG. 13, in either case, G_4N was found to be able keep DNA from interacting with Sp1-167D protein. When only the DNA binding domain of Sp1 alone was tested, G_4N appeared to be more efficient in displacement of the bound Sp1 than blocking Sp1 from binding to the enhancer, as shown by the gel mobility-shift analysis (~~Figure 13, A,B,D~~FIGS. 13A, B, and D). We have also examined whether the bound G_4N can be replaced by Sp1-167D. In this study, the inhibition of Sp1-167D

binding by G_4N was first established by the mobility-shift analysis (~~Fig~~ FIG. 13C, lanes 2 and 5). When the G_4N bound template was challenged with additional Sp1-167D, we observed a dosage dependent increase of the band intensities of the Sp1-167D/DNA complex (~~Fig 6C~~FIG. 13C, Lanes 6,7) indicating the displacement of G_4N by Sp1-167D from the template.

Example 13

Inhibition of Sp1 Regulated Tat-Transactivation of HIV Promoter Activity by G_4N.

As reported previously, methylated NDGA derivatives can block Sp1 binding to the enhancer sites of a variety of viral promoters including HIV, ICP4 of HSV, E_6/E_7 gene of HPV (8, 9, 10). We further tested the G_4N effect on the Tat-transactivation of HIV promoter activity in Cos cells by the SEAP assay as previously described. Basal level of the HIV LTR driven SEAP expression was previously found to be barely detectable in Cos cells. There were 60-fold or more increase in SEAP expression when Cos cells were cotransfected with the CMV promoter driven Tat gene (8). Such Tat-driven ransactivation of the HIV LTR promoter activity was previously shown to be Sp1 regulated (7, 8). In the presence of G_4N, we observed inhibition of HIV transactivation in a dose-dependent fashion (~~Figure~~FIG. 14). An average value IC_{50} value of 36 μM for G_4N was comparable to that of 3-O-methyl NDGA, Ma1.4 (IC_{50} 25 μM) and somewhat higher than that of tetra-methyl NDGA, M_4N (IC_{50} 11 μM). The differences perhaps are due to the chemical nature of the test compounds affecting the drug uptake to the cells.

Example 14

Inhibition of SIV-1 and HIV-1 Production in Cell Cultures by G_4N.

Both HIV-1 and SIV are retroviruses that require integration into the host genome to complete their replication. Both rely on host transcription factors for their proviral transcriptions. Sp1 plays a central role for such expression in these two viruses sharing an almost identical mode of transcription regulation. In anticipation of using SIV infected rhesus monkeys as animal model for testing the antiviral effect of G_4N, we have studied

and compared the G_4N effect in inhibition of SIV in 174 x CEM cells with that of HIV in H9 cells. Cellular toxicities of G_4N in these two cell lines were also examined. For SIV inhibition study, 10^7 174 x CEM cells were mixed with high titer stock of SIVmac 239 at 37°C for two hours and then washed twice with cold PBS buffer to remove the unabsorbed virus. Cell suspension was aliquoted into each well of three 96 well plates. Various concentrations of the G_4N solutions were made from freshly prepared stock and aliquoted separately and each to six wells in a column of one 96 well plate. Culture ~~supernantants~~ supernatants were collected every four days post infection. (P.I.) and fresh medium containing appropriate concentrations of the drug were added to the culture following supernatant collections. Viral production was assayed by a modified p27 core antigen capture ELISA as shown (~~Figure~~FIG. 15). There was no SIV production detected using G_4N in concentrations above 5 CIM. At G_4N concentrations below 2.5 CIM, SIV production was detected (~~Fig.~~ FIG. 15) in culture supernatants from 4^{th} and 8^{th} days post infected cultures as compared to viral production in the absence of the drug. G_4N (250 μM or less) showed no toxic effect on uninfected 174 x CEM cells, as determined by the MTT assay (34).

A similar experiment was also carried out for the study of inhibition of HIV-1 by G_4N in H9 cells. The H9 cells were subcultured at 1 x 10^5/ml and were infected with an AZT resistant strain of HIV-1(HIV-1RTMF). G_4N in different concentrations was added two hours after infection. Fresh medium change was made every four days. Cell growth in the presence of G_4N was monitored carefully during the nine-day experimental period. Viral production was assayed by a p24 core antigen capture ELISA. As shown (~~Figure~~FIG.16), G_4N concentration of 80 CIM completely inhibited HIV replication in H9 cells. An IC_{50} of 12 μM CIM G_4N for the inhibition of HIV-1 RTMF was found. Again, there was no detectable toxicity to uninfected H9 cells within the range of the assay (and below 250 μM).--

At page 36, please replace the text beginning on line 11 to the bottom of the page with the following:

--To determine whether the Sp1-regulated survivin gene expression in C3 cells is reduced by M_4N treatment, we treated the cells with 40 μM M_4N for 24 hours and 72 hours. As shown in ~~Figure~~FIG. 17, treatment of cells with M_4N resulted in a significant decrease in survivin gene expression in a time-dependent manner. Treatment with 40 μM M_4N for 24 hours and 72 hours resulted in 65% and 80% reduction in survivin expression, respectively. Untreated cells did not show any reduction in survivin gene expression.

Survivin protein was also shown by immunoblotting to be downregulated by 72 hours of M_4N treatment. This downregulation was dosage-dependent (~~Figure 2~~FIG.18).

Example 16

Induction of Apoptosis with M_4N treatment

Because our data showed that M_4N resulted in survivin mRNA and protein reduction, we investigated whether this reduction induces apoptosis since survivin has anti-apoptosis function. As shown by immunoblotting of caspase-3 (~~Figure 3~~FIG. 19), treatment of M_4N for 72 hours resulted in caspase-3 activation. This activation would be expected to result in an increase in an increase in apoptosis in cells treated with M_4N.--